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## Plant Defense Peptides

**Abstract:** Eight families of antimicrobial peptides, ranging in size from 2 to 9 kD, have been identified in plants. These are thionins, defensins, so-called lipid transfer proteins, hevein- and knottin-like peptides, MBP1, IbAMP, and the recently reported snakins. All of them have compact structures that are stabilized by 2–6 disulfide bridges. They are part of both permanent and inducible defense barriers. Transgenic overexpression of the corresponding genes leads to enhanced tolerance to pathogens, and peptide-sensitive pathogen mutants have reduced virulence.

**Keywords:** antimicrobial peptides; plant defense peptides; thionin; defensin; lipid transfer protein; hevein- and knottin-like peptides; MBP1; IbAMP; snakin; disulfide bridge; pathogen

### INTRODUCTION

It is becoming increasingly evident that, despite their distinct modes of life, plants and animals share some common elements in their mechanisms of defense against pathogens. The initial, naive view of a non-specific system of active and passive defense in plants and a specific immune system in animals has been superseded by the recognition that plants also have a specific nonself-surveillance system and that animals have a nonspecific system for innate immunity, besides the specific adaptive immune system (for review, see Refs. 1–9).

Antimicrobial peptides have been long considered to play a key role in plant defense, both as part of preexisting, developmentally regulated defense barriers

and as components of the defense responses induced upon infection. Demonstration of a possible defense role for a given type of peptide involve observations of diverse nature, none of which can be conclusive by itself. Some relevant criteria are the following: (a) antimicrobial activity *in vitro*; (b) gene expression, peptide distribution, and peptide concentrations *in planta* (before or after infection) that are congruent with a defense role; (c) correlation of the variation of expression levels (natural or genetically engineered) with the severity of symptoms; (d) correlation of the variation of the pathogen resistance to plant peptides (natural or genetically engineered) with virulence. Considerable progress has been recently made in the identification of plant antimicrobial pep-

tides and in their investigation according to these criteria.

Antimicrobial peptides from animals may be linear or form complex globular structures in which antiparallel  $\beta$ -sheets are stabilized by disulfide bonds, whereas in plants, only disulfide-bonded peptides of the second type have been identified so far.<sup>1-9</sup> Among plant antimicrobial peptides, thionins were the first whose activity against plant pathogens was demonstrated in vitro.<sup>1,10</sup> Subsequently, several families of cysteine-rich peptides have been characterized, including defensins,<sup>8,9</sup> lipid transfer proteins<sup>6,8,9</sup> (LTPs), hevein-type peptides,<sup>8</sup> knottin-type peptides,<sup>8</sup> and others. In this review, we summarize recent advances concerning the structural and functional properties of all these families of putative defense peptides from plants.

## THONINS

### Structure and Distribution

The name "thionins" has been proposed to designate a family of homologous peptides that includes purothionins, which were first isolated from wheat seeds,<sup>11</sup> and their homologues from various taxa, such as viscotoxins, phoratoxins, and crambins (see Ref. 12).

The original purothionin from hexaploid wheat was later found to be heterogeneous,<sup>13-15</sup> and in a survey of endosperm thionins in 22 diploid, tetraploid, and hexaploid species of the *Aegilops-Triticum* group, the presence of at least one variant per diploid genome complement was demonstrated.<sup>16</sup> Two thionins from barley endosperm<sup>17-20</sup> designated  $\alpha$ - and  $\beta$ -hordothionin and two from oats endosperm (*Avena sativa*) have been also characterized.<sup>21</sup>

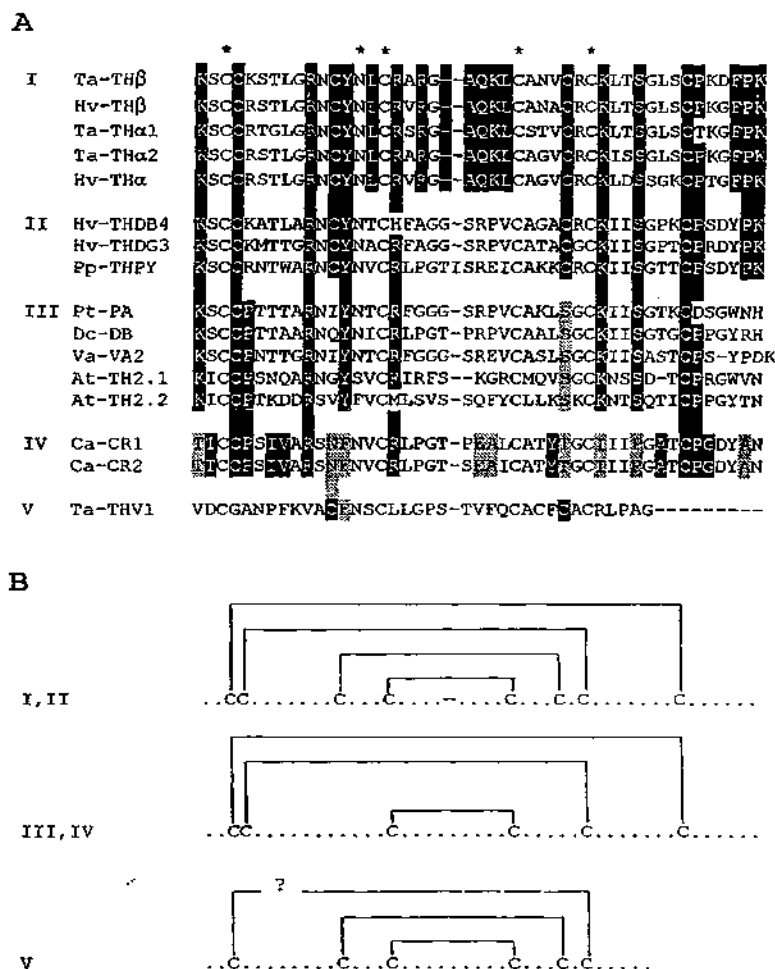
The viscotoxin from leaves and stems of European mistletoe (*Viscum album*, Loranthaceae) was also found to be a mixture of closely related components. Similar toxins, such as phoratoxins A and B from *Phoradendron tomentosum*, denclatoxin B from *Dendrophthora clavata*, and ligatoxin A from *Phoradendron liga*, have been also identified within the Loranthaceae (reviewed in Ref. 12). Additional leaf thionins have been identified in *Pyrularia pubera*, a parasitic plant from the Santalaceae,<sup>22</sup> and in barley.<sup>23,24</sup> The crambin reported by Van Etten and co-workers<sup>25</sup> was also found to be a mixture of two variants, whose primary structures were homologous to the thionins and the viscotoxins.<sup>26,27</sup>

The mature thionin peptides are generally 45-47 amino acids in length. The available amino acid sequences from the thionins (either directly determined

or deduced from cDNAs) can be classified into at least five types (I-V), one of which (type V) lacks the C-terminal nonapeptide.<sup>12-28</sup> The original purothionin isolated from wheat endosperm<sup>11</sup> has four disulfide bridges and is highly basic, with no negatively charged residues. Known sequences of this type (I) comprise 45 amino acid residues, 8 of which are in the central disulfide loop. Type II thionins have been isolated from the leaves of *Pyrularia pubera*<sup>22</sup> and of barley,<sup>23,24</sup> and have four disulfide bridges at the same positions as those of type I, but the molecules are less basic, with some negatively charged residues, and their central disulfide loop contains one or two more amino acid residues than the type I. The third type (III) includes the viscotoxins and phoratoxins from mistletoes (Loranthaceae), and has three disulfide bridges conserved with respect to the previous types; they contain fewer basic amino acid residues; and their sequence has 46 residues, 9 of which are in the central disulfide loop. The crambins isolated from the Abyssinian cabbage (Cruciferae) represent the fourth type (IV), which has the same sequence length and disulfide-bridge arrangement as type II thionins, but the molecules are neutral, with a low proportion of charged amino acid residues. Type V is quite divergent: the second and eighth cysteines of type I thionins are missing through point mutation and deletion, respectively, thus disrupting the first and second disulfide bridges and potentially allowing the formation of a new bridge between the unpaired cysteines. Type V thionins are also neutral and have been identified as cDNAs from developing kernels of wheat and *Aegilops* species.<sup>28,29</sup>

Based on the disulfide-bond structure, all known thionins can be classified into three groups: a group with 4 disulfide bonds, which would include types I and II, a group with only 3 of the above disulfide bonds (types III and IV), and a group that presumably has only 2 of the above bonds, plus a novel one (Figure 1).

The three-dimensional structure of thionins has been studied in detail, both in crystals and in solution, and they have become model molecules in the development of new methods of structure elucidation.<sup>30,32</sup> It has been shown that thionins of types I, III, and IV, in spite of their extreme divergence, have essentially the same three-dimensional shape, which resembles the Greek capital letter gamma ( $\Gamma$ ). The molecules are amphipathic, with a rigid structure, and present very similar three-dimensional shapes in solution and in crystal form. The long arm is formed by two antiparallel  $\alpha$ -helices and the short arm by a  $\beta$ -sheet consisting of two short antiparallel  $\beta$ -strands. The hydrophobic residues are clustered at the outer surface of



**FIGURE 1** Thionin types. (A) Alignment of representative amino acid sequences of the different types (I-V). (B) Disulfide bridge structures of the different types. (\*) Conserved positions.

the long arm of the  $\Gamma$ , whereas hydrophilic residues mainly occur at its inner surface and at the outer surface of the corner of the  $\Gamma$ .<sup>30,32</sup> It has been proposed that type I thionins have a binding site for phospholipids, which may be implicated in their toxic activity.<sup>33,34</sup>

The evolution of thionins, particularly that leading to changes in the number of disulfide bridges, merits special attention because it represents a general evolutionary problem. Divergence between types I and V seems to have occurred through accelerated evolution, a process that has affected the amino acid sequence of the mature thionin but not the precursor domains corresponding to the N-terminal signal peptide and the long C-terminal acidic peptide described in the next section.<sup>28,29</sup> This process involved a deletion and a nonsynonymous nucleotide substitution rate equal to the synonymous substitution rate in the thionin sequence. The coding sequences of two type V thionins have been absolutely conserved during the approxi-

mately 10,000 years that the D genomes of the diploid and the allopolyploid *Triticum* and *Aegilops* species have been evolving separately, whereas the introns have diverged, especially the larger one, which has suffered one major and several minor deletions.

Percentages of divergence between types I and V in the mature-protein domain (59–69%) are about twice as high as those occurring in the other domains, namely signal peptide, C-terminal acidic peptide, and introns (21–36%). In contrast, divergence in the mature protein domain of type V precursors is equal to or lower than within this domain of type I thionins, and is certainly much lower than in the corresponding introns.<sup>28,29</sup> Type I thionins have four disulfide bridges, whereas those of type V have only three. It is possible that a temporary loss of function due to mutation of one cysteine (gain or loss) in the duplicated gene might have resulted in a period of accelerated evolution. Mutation of a second cysteine (loss or gain) would have then led to a mature thionin with

an even number of cysteines (a common feature of all known thionins) and to a recovery of function that would in turn impose a slower rate of evolution.<sup>29</sup>

## Molecular Biology

Barley endosperm thionins are synthesized by membrane-bound polysomes as much larger precursors that undergo at least two processing steps.<sup>18,19,35</sup> The deduced structures of these precursors consisted of an N-terminal signal peptide, followed by the mature protein and a C-terminal acidic protein. The same precursor structure was later found for thionins of the other types,<sup>23,24,28,29,36,37</sup> which strongly suggests that all types of thionins have the same biosynthetic pathway.

Cellular fractionation studies and electron microscopy of developing barley endosperm have shown that type I thionins are in the particulate fraction, associated with electron-dense ovoidal structures in the periphery of protein bodies.<sup>35,38</sup> A recent report<sup>39</sup> has shown that in barley leaves, mature type II thionins accumulate inside vacuoles. Both purified vacuoles and an acid (pH 5.5) extract from leaves were able to process the precursor and excise the acidic peptide. Processing by both lysed vacuoles and by the purified proteinase was inhibited by  $Zn^{2+}$  and by  $Cu^{2+}$ , but not by inhibitors of previously described vacuolar processing thiol or aspartic proteinases. Variants of a fusion protein with altered processing sites that represented those of thionin precursors from different taxa were readily processed by the proteinase, whereas changing the polarity of either the C-terminal or N-terminal residues of the processing site prevented cleavage by the enzyme.<sup>39</sup>

Using aneuploids, genes encoding thionins have been associated with specific chromosomes of wheat and related species.<sup>20,28,29,40-43</sup> The gene for  $\alpha$ -hor-dothionin, a type I thionin from barley endosperm, has two introns, 420 and 91 nucleotides long, that interrupt the sequence encoding the C-terminal, acidic peptide of the precursor.<sup>20</sup> Genomic clones of type II thionins have two introns in similar positions as those of type I clones.<sup>43</sup>

The expression of type II thionin genes has been investigated in barley leaves and a number of interesting responses of these genes to external stimuli have been described. Large amounts of messenger for type II thionins were detected in dark-grown barley seedlings.<sup>23,24</sup> Steady state messenger levels seemed to be higher in the basal  $\frac{1}{3}$  of the leaf (younger cells) than in the apical  $\frac{2}{3}$  (older cells), and to decline sharply upon illumination.<sup>24</sup> The effect of light has been further investigated by Reimann-Philipp et al.,<sup>44</sup> who

have postulated the mediation of two photoreceptors, phytochrome-a and a blue-light-absorbing photoreceptor. Synthesis of thionins ceased upon illumination, but the previously accumulated thionin was rather stable.<sup>44</sup> The inhibitory effect of light can be overcome by stress- and pathogen-induced signals. It has been shown that fungal infection induces a transient expression of the thionin genes in the leaves<sup>43,45</sup> and that the chlorides of divalent cations ( $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Zn^{2+}$ ) elicit a more permanent response.<sup>46</sup>

## Biological Properties

Thionin from wheat endosperm could substitute for thioredoxin *f* from spinach chloroplasts in the dithiothreitol-linked activation of chloroplast fructose-1,6-bisphosphatase.<sup>47</sup> This led to experiments suggesting a possible role of thionins as secondary thiol messengers in the redox regulation of enzymes. An activity of thionins that might be related to their redox properties is the ability to form selective disulfide bridges with other proteins.<sup>48,49</sup> The enzymes  $\beta$ -glucuronidase and neomycin phosphotransferase II were inhibited by thionins through the formation of disulfide-linked adducts and the inhibition was reversed by DTT.<sup>48,49</sup>

The ability of thionins to induce leakage of intracellular material was first demonstrated in bacteria and in yeast. The effect could be reversed by certain divalent cations, such as  $Ca^{2+}$ ,  $Zn^{2+}$ , or  $Fe^{2+}$  (reviewed in Ref. 12). The cytotoxic effects of thionins of types I and IV on cultured mammalian cells occurred at the minimum concentration that caused leakage of  $Rb^{1+}$  and of uridine.<sup>50</sup> Concentrations of thionins that had no detectable effects on the cultured cells lead to inhibition of translation by antibiotics such as hygromycin B that do not normally cross the plasma membrane.<sup>50</sup> The effect of thionins on fungal membranes has been investigated in *Neurospora crassa*, where the minimum concentration required to cause leakage and growth inhibition were similar.<sup>51,52</sup> The effects of thionins on smooth-muscle contraction and on insect flight muscle, as well as the sensitivity to thionins of A31 cells infected with the Moloney strain of murine leukemia virus, are all probably related to interactions of thionins with the cell membrane (reviewed in Ref. 12). It has been recently reported that thionins induce leakage and aggregation of artificial, negatively charged membranes under conditions in which other plant toxic peptides have no effect.<sup>53,54</sup>

The toxicity of thionins to plant pathogens was first reported by Fernandez de Caleyra et al.,<sup>10</sup> and it has

been extensively investigated.<sup>43,55-59</sup> Type I thionins were also found to be toxic to mice, guinea pigs, and rabbits when injected intravenously or intraperitoneally, but not upon oral administration.<sup>60</sup> Type III thionins, isolated from the leaves of the mistletoes and related species, were also found to be toxic upon parenteral administration to mice and cats (see Ref. 61). At sublethal doses they produced hypotension and bradycardia, and had a negative inotropic effect on the heart muscle. Intraarterial administration, in higher doses, produced vasoconstriction in arteries of skin and skeletal muscle.<sup>61</sup> Cytotoxic effects on cultured mammalian cells have been reported for different thionin types.<sup>22,50</sup>

The hypothesis that thionins might play a role in the protection of plants against pathogens was proposed by Fernandez de Caleya et al.,<sup>10</sup> who investigated the susceptibility to wheat endosperm thionins of phytopathogenic bacteria in the genera *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Erwinia*, and *Corynebacterium*. Purified genetic variants of these thionins differed in activity and showed some degree of specificity. Both endosperm (type I) and leaf (type II) thionins from barley inhibit the fungi *Thielaviopsis paradoxa*, a pathogen of sugar cane, and *Drechslera teres*, a pathogen of barley, at concentrations of  $5 \times 10^{-4} M$ .<sup>43</sup> Fungal and bacterial pathogens included in a recent survey were inhibited by thionins at concentrations in the  $10^{-6}$ – $10^{-5} M$  range, which are similar to those found in certain plant tissues.<sup>57</sup>

Recent experiments *in planta* are also indicative of a defense role for the thionins. Thionin mRNA is transiently induced in barley upon infection with *Erysiphe graminis* in both susceptible and resistant cultivars.<sup>43,44</sup> Transgenic expression in tobacco plants of a barley thionin gene showed reduced lesion size when the plants were challenged with two strains of *Pseudomonas syringae*,<sup>62</sup> whereas other strains did not seem to be affected.<sup>56</sup> More recently, overexpression of an endogenous thionin has been reported to enhance resistance of *Arabidopsis thaliana* against *Fusarium oxysporum*<sup>63</sup> and *Plasmidiophora brassicae*.<sup>64</sup> A significant observation in support of a defense role for thionins is the fact that thionin-sensitive mutants of *Ralstonia solanacearum* were found to be avirulent by Titarenko et al.<sup>65</sup>

## DEFENSINS

### Structure and Distribution

Plant defensins are a family of antimicrobial peptides, 45–54 amino acid residues in length, that have

been isolated from different taxa and are probably ubiquitous in the plant kingdom (for reviews, see Refs. 8 and 9). Based on their amino acid sequences, four different defensin groups or subfamilies can be established,<sup>8,66-70</sup> and as discussed later, the structural differences seem to correlate with changes in antimicrobial specificity (Figure 2). All known members of this family have eight disulfide-linked cysteines, including one at the C-terminus. Apart from these eight cysteines, there are a few other residues that are highly conserved in all types (Figure 2), whereas there are residues that are conserved in two or more types.

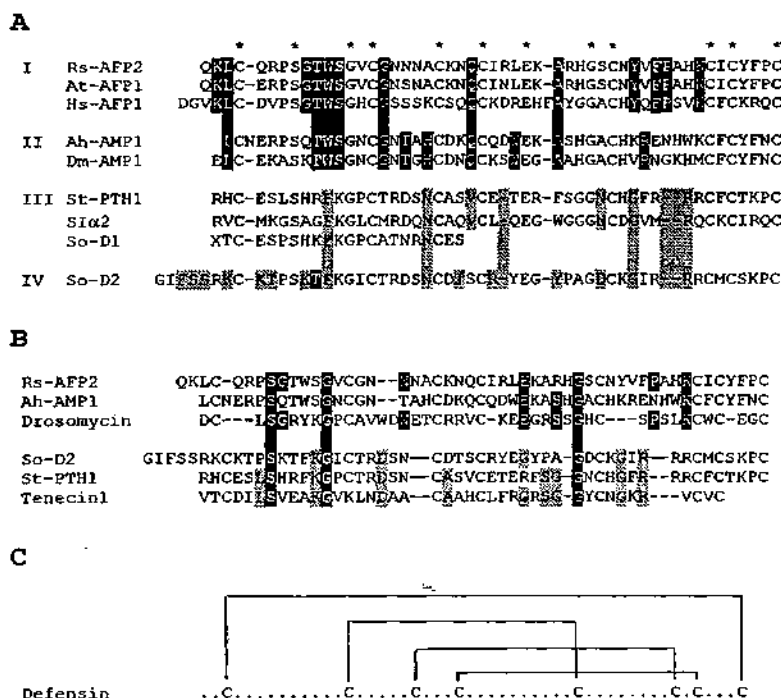
Similarities of plant defensins with respect to those of insects are weak but discernible at the level of the primary structure (Figure 2B)—types I and II being closer to drosomycin from *Drosophila melanogaster*<sup>71</sup> and groups III and IV to tenecin from *Tenebrio molitor*<sup>72</sup>—and striking at the three-dimensional level.<sup>69,73-76</sup> The three-dimensional structure of plant defensins consists of a triple-stranded antiparallel  $\beta$ -sheets and one  $\alpha$ -helix that is stabilized by disulfide bonds: all these domains, except the N-terminal  $\beta$ -strand, are conserved in the insect defensins.<sup>8</sup>

The distribution of defensins in the plant is consistent with their putative defense role. Thus they have been identified in leaves,<sup>77,78</sup> tubers,<sup>79</sup> flowers,<sup>77,79-81</sup> pods,<sup>82</sup> and seeds.<sup>66,83,84</sup> In *Arabidopsis* there are at least 5 different defensins (identified as ESTs) whose genes are expressed in a tissue-specific manner.<sup>68</sup> Plant defensins are preferentially located in peripheral cell layers and have also been reported in the xylem, in stomatal cells and in cells that line the substomatal cavity, all of which are locations where first contact and entry of pathogens take place.<sup>8,79</sup>

### Molecular Biology

Most of the known plant defensins have typical signal peptides and lack a propeptide,<sup>78,81,82,85,86</sup> but in two cases, a 30-residue C-terminal propeptide, similar to that in thionin precursors, has been reported.<sup>80,87</sup> The significance of this different precursor structure remains to be elucidated, as is the case with thionins.

Expression of some defensin genes is developmentally regulated in a rather strict manner,<sup>68</sup> whereas that of others is greatly influenced by abiotic and biotic external stimuli.<sup>8,77</sup> Thus, defensin genes induced upon pathogen infection have been identified in pea,<sup>82</sup> tobacco,<sup>80</sup> radish, and *Arabidopsis*.<sup>8,88</sup> At least in some of these cases, the induction is systemic, as can be detected in noninfected leaves of the infected plant.<sup>8,78,88</sup> A recent report indicates that, in *Arabidopsis* seedlings, a defensin gene type is inducible by



**FIGURE 2** Defensin types. (A) Alignment of representative amino acid sequences of the different types (I–IV). (B) Alignment with insect defensins drosomycin (types I and II) and tenecin (types III and IV). (C) Disulfide bridge structure. (\*) Conserved positions.

methyl jasmonate and silver nitrate, as well as by phytopathogenic fungi, such as *Fusarium oxysporum* f. sp. *Matthiolae*, in a similar manner as a thionin gene, whereas other defensin genes are not affected by the same treatments.<sup>8</sup>

## Biological Properties

Although inhibition of  $\alpha$ -amylases at high inhibitor concentrations has been reported for some cereal defensins,<sup>8</sup> the ability to inhibit bacteria and fungi is their most relevant biological property. The antimicrobial activity of defensins was first reported for two isoforms isolated from radish seeds<sup>83</sup> and now have been extensively studied in defensins from different species. Based on these properties, four defensin groups can be made that essentially match the structural subfamilies.<sup>66,67,70,89</sup> Defensins of group I cause inhibition of gram-positive bacteria and fungi, and the fungal inhibition occurs with marked morphological distortions of hyphae (branching); those of group II are active against fungi, without induction of hyphal branching, and inactive against bacteria; those of group III are active against gram-positive and gram-negative bacteria, but inactive against fungi; and those of group IV are active against gram-positive and

gram-negative bacteria, and against fungi, without causing hyphal branching.

The antifungal activity of plant defensins is antagonized by increasing the ionic strength and, most notably, by divalent cations.<sup>8,66,83,84</sup> The magnitude of this effect depends both on the fungus and the plant defensin type.<sup>8</sup> No toxicity of plant defensins to animal or plant cells has been found.<sup>8,83</sup> The mechanism of action of defensins is not yet completely understood,<sup>8</sup> although it has been shown that they mediate a sustained  $\text{Ca}^{2+}$  influx and  $\text{K}^{+}$  efflux, when added to hyphae of the fungus *Neurospora crassa*, and that, in contrast with thionins, they do not cause permeabilization to isoaminobutyric acid or have any effect on electrical currents in artificial membranes.<sup>90</sup> These observations imply that fungal growth is not inhibited by direct peptide–lipid interaction.<sup>8</sup> A recent mutational analysis of defensin Rs-AFP2 from radish has revealed two adjacent sites in its sequence that are important for antifungal activity: Tyr-38, Phe-40, Pro-41, Ala-42, Lys 44, and Ile-46 appear clustered in the three-dimensional model to form the first site, whereas Thr-10, Ser-12, Leu-28, and Phe-49 would form the second site.<sup>67</sup> However, out of the 11 positions at which a mutational change produced a significant decrease in the activity of this peptide, only 2 are

A

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Hv-LTP2  AITCGVSSALGPCAFAK--ESGTSFSAAGCCSEVKR--AGLARSTAKQAT--RELKSVAGAY---NAGRAAGI--SRGGVSVPTISASVD--SKIH
Hv-LTP4  AISCQVSSALSPCISAR--ENGAKPPAAC--SEVKR--AGAAQSTADKQAACKIKSA--GGL---NAGKAGI--SMGGVSVPTISASVD--SKIR
Zm-LTP   ALSCGVASAIAPCISAR--QGGSGPSAGCCSEVRS--NNAA--RTTADRRAN--NLKNA--AGVSG--NAGNA--SI--SKGGVSVPTISTD--SRVNN
Nt-LTP   ALTCCVYTAGLAPCLPYLQ--ERGP--LGCCCGEVKN--LGS--KTTADKTA--CLKSA--NAIKGI--DLNK--AGI--SVSK--NI--PVKIS--PSTD--STVQ
So-LTP   GITCGVSSKLAPOCIGYLO--SGP--LGCCCGCHIKANAA--ATTEDKDA--NLKSA--NAIKGI--NYGKA--AGI--GMGG--HI--PVATIS--PSTN--LNAVH
Hv-LTP   ALNCCGVSSKMKPCLTYVQ--SGP--GPSGEC--GVNR--HNQ--QSSG--DRTV--LCLKGI--RGHNL--NLNNA--SI--SKKN--NVPTIS--PDID--SRIV
Ta-LTP   -IDCGHVDLVRPCLSYVQ--SGP--GPSGQCC--GVNR--HNQ--ARSQ--DRTV--LCLKGI--RGHNL--NEDNA--SI--EPK--GN--LVPTIS--LNID--SRV
At-LTP1  ALSGGSVNSNLAAGIGYVLO--GV--IPPA--CGSV--KNSI--AKTT--PDQQA--NCG--GA--RALG--SGLN--AGI--KAG--CH--I--PVKIS--PSTN--SKTVR
Gh-LTP6  AISYDQVKSLLPCVGVVRGNARPAPPNYCK--IRSLKSA--RIRL--DQAA--CKIKSL--ADISDI--NYGVA--AGL--GG--NI--HI--PVKIS--PSTD--SKRVK
Ace-AMP1 QNIEPRNRIVTEGAYGL--ERA--PIA--CC--RALND--LRFV--NTRN--LR--AA--RL--LVGV--VNRN--PGLRN--PRFQ--NI--RD--RNT--FVR--PFW--PRIC--GRIN

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B

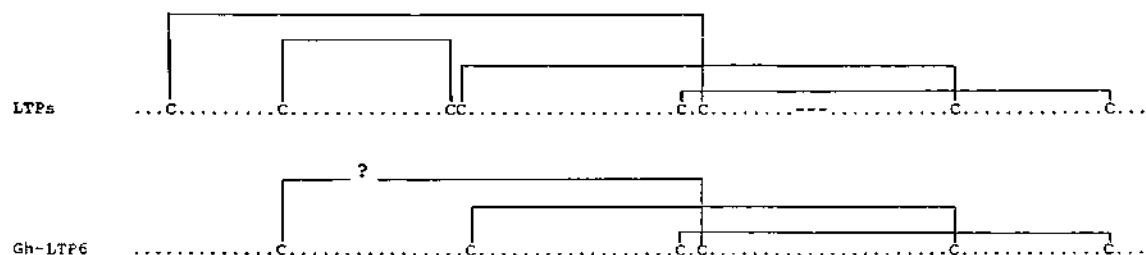


FIGURE 3 Lipid transfer proteins (LTPs). (A) Alignment of representative amino acid sequences. (B) Disulfide bridge structures.

conserved in defensin So-D2, which is fully active, and, particularly the change Tyr → Gly at position 38 inactivated Rs-AFP2, whereas Gly is at that position in So-D2.<sup>70</sup> It is possible that different mechanisms of action operate among the different defensins. Indeed, synthetic 15-mer peptides comprising the region from Cys-27 to Cys-47 of defensin Rs-AFP2 were active against fungi, but only one of them was active against bacteria.<sup>89</sup>

Besides the response of some defensin genes to pathogen infection, a number of observations *in planta* support their putative defense role. Experiments with radish seeds have demonstrated that defensins represent over 30% of the proteins released during germination (about 1 μg/seed) and that the released defensin is sufficient for fungal inhibition, an effect that may contribute to the enhancement of seedling survival rate.<sup>78</sup> Additionally, transgenic expression in tobacco of the Rs-AFP2 defensin from radish (up to 0.2% of leaf proteins) resulted in a sevenfold reduction in lesion size with respect to the nontransformed control, upon infection with the foliar fungal pathogen *Alternaria longipes*.<sup>78</sup>

## LIPID TRANSFER PROTEINS

### Structure and Distribution

The so-called nonspecific lipid transfer proteins (LTPs) are a family of peptides previously thought to be involved in lipid shuttling between organelles<sup>91</sup>

and have been recently implicated in plant defense.<sup>92-96</sup> Plant LTPs are 90-95 amino acid polypeptides that have been identified (at the protein and/or cDNA levels) in various tissues from a high number of mono- and dicotyledonous species.<sup>6,91</sup> They were initially reported in spinach leaves, maize coleoptiles, and barley aleurone,<sup>91,97</sup> and later found to be distributed throughout the plant, externally associated with the cell wall and cuticle of epidermal and peripheral cell layers.<sup>93,94,98,99</sup>

Comparison of reported amino acid sequences (directly determined or deduced from nucleotide sequences) indicates that about  $\frac{1}{3}$  of the residues are conserved, including the 8 disulfide-linked cysteines (Figure 3). Only recently, an LTP with 6 cysteines has been reported in cotton fiber.<sup>100,101</sup> The two missing cysteines in this LTP would correspond to two different disulfide bonds of the known LTP disulfide pattern.

Tertiary structure of LTPs has been extensively studied, both by x-ray diffraction and proton nmr,<sup>102-106</sup> and structural models of their interaction with lipids have been proposed.<sup>104,106-108</sup> Both as crystal and in solution, the protein has a globular structure that consists of a bundle of four α-helices linked by flexible loops with a hydrophobic cavity that may accommodate a variety of lipids.<sup>104-108</sup>

### Molecular Biology

The cytoplasmic role originally proposed for plant LTPs, shuttling lipids between the endoplasmic retic-

ulum and organelle membranes, seems unlikely because it is now known that they are synthesized as precursors with typical signal peptides,<sup>95,109-111</sup> secreted in cell culture,<sup>112,113</sup> and externally associated with the cell wall.<sup>94-97,100,101</sup> The only known exception seems to be an LTP-like peptide from onion seeds that encodes a short propeptide, 12-residue long, at the C-terminus of the mature LTP, besides a typical N-terminal signal peptide.<sup>114</sup>

Plant LTPs were initially thought to be encoded by one or two genes per haploid genome whose expression was developmentally regulated and highly restricted to special locations.<sup>115-118</sup> However, it was later found that they are encoded by divergent multi-gene families and ubiquitously expressed in the plant, especially over exposed surfaces and in vascular tissues.<sup>92-96,119</sup> For example, at least six genes (on chromosomes 3H, 5H, and 7H) have been identified in barley.<sup>95</sup> Overlapping expression of some of these genes (*Hv-LTP2-4*) has been detected in stem, shoot apex, leaves, spike, kernel, and roots,<sup>95</sup> while expression of one of them (*Hv-LTP1*) was restricted to the aleurone cell layer.<sup>112</sup> The highest LTP expression levels have been generally observed in epidermal or peripheral cell layers surrounding the different organs.<sup>95,97,99,116,118,120,121</sup> A substantial amount of the barley LTPs could be washed from leaves by simple imbibition in an aqueous buffer,<sup>6,122</sup> and in broccoli leaves, an LTP was found to be the main protein of the wax layer.<sup>98</sup>

Apart from the developmentally regulated expression of LTP genes, it has been shown that these genes respond to pathogen infection in a complex manner.<sup>6,95,122</sup> Thus, LTP genes can be induced above basal levels or be switched off by different plant pathogens that infect barley<sup>6</sup>: infection by the fungus *Erysiphe graminis* similarly induces the genes above basal levels (and with the same timing) both in the compatible and in the incompatible interaction; infection by the fungal pathogen *Rhynchosporium secalis* increases LTP gene expression only in the incompatible interaction, not in the compatible one, and this induction is under the control of a resistance gene (*Rht3*); and infection with the compatible bacterial pathogen *Pseudomonas syringae* pv *japonica* switches off LTP gene expression. These observations are congruent with the proposed defense role of LTPs and are similar to those made for other defense peptides.

Based on the observed preferential location of LTPs, it has been proposed that they may be involved in the deposition of cutin or of other lipophilic substances,<sup>97</sup> but this would be inconsistent with their distribution in stems and vascular tissues.<sup>95,118</sup> In any

case, as has been previously indicated,<sup>6,8</sup> a defense role is not incompatible with other functions.

## Biological Properties

Antimicrobial activity of LTPs has been reported for all members of the family tested, including those isolated from barley, maize, spinach, arabidopsis, radish, and broccoli.<sup>92,99</sup> The relative activities of different LTPs vary between pathogens, suggesting that they have some degree of specificity.<sup>6,93</sup> While certain LTPs were much more active than thionins against the bacterial pathogen *Clavibacter michiganensis* subsp. *sepedonicus*, the opposite was true against the fungus *Fusarium solani*, indicating that the two families of antimicrobial peptides might complement each other when simultaneously present in a tissue.<sup>6,93</sup> Furthermore, synergism between the two types of peptides was observed in the case of the bacterium, whereas the activity against the fungus was merely additive.<sup>6,93</sup>

The possible defense role of LTPs is supported by the observation that transgenic tobacco and *Arabidopsis* plants overexpressing a barley LTP showed drastic reduction of disease symptoms after infection of the leaves with the bacterial pathogen *Pseudomonas syringae*.<sup>123</sup>

Different strains of given pathogens showed different susceptibility to a given LTP,<sup>122</sup> which indicated that resistance/susceptibility of the pathogen toward a plant peptide might be relevant in the outcome of a plant-pathogen interaction. Indeed, mutants of the pathogen *Ralstonia solanacearum* that were more sensitive to LTPs than the wild type were found to be completely avirulent,<sup>65</sup> a finding that further supported a defense role for LTPs.

## OTHER PEPTIDE FAMILIES FROM PLANTS

### Hevein- and Knottin-Like Peptides

Hevein, the most abundant protein in the latex of rubber trees, is a 43-residue, cysteine-rich peptide homologous to the chitin-binding domain of different types of multidomain proteins from plants,<sup>8,124,125</sup> and to other single-domain peptides that have antimicrobial properties.<sup>126-128</sup> The hevein-like, antimicrobial peptide from sweet pepper contains the same 8 disulfide-linked cysteines that are present in the peptide from latex,<sup>126,128</sup> whereas in that from amaranth, the last two cysteines are missing because of a C-terminal deletion.<sup>127</sup> Three  $\beta$ -sheet strands, as well as



an  $\alpha$ -helix turn that links the second and third strands, are the most relevant three-dimensional features of the hevein structure, as determined by proton nmr.<sup>129</sup>

Antimicrobial peptides, 36–37 amino acid residues in length, have been isolated from seeds of *Mirabilis jalapa*.<sup>130</sup> These peptides have 6 disulfide-linked cysteines that form the so-called knottin pattern, which resembles that of the distantly related hevein-like peptides.<sup>131–134</sup> Chitin-binding, hevein-like antimicrobial peptides also have been reported from seeds of *Pharbitis nil*<sup>135</sup> and from sugar-beet leaves.<sup>136</sup>

The amino acid sequence deduced from a cDNA encoding hevein includes an N-terminal signal peptide and a C-terminal propeptide that is homologous to pathogenesis-related protein PR4 from tobacco.<sup>124,134</sup> The cDNA from the amaranth hevein-like protein encodes a similar precursor, but the C-terminal peptide is shorter.<sup>137</sup> Knottin-type precursors do not include a C-terminal propeptide,<sup>138</sup> though, as is the case for the hevein type, the peptides are exported to the apoplast.<sup>139</sup>

Both hevein- and knottin-like peptides inhibit a wide range of fungi and gram-positive bacteria in vitro, and their activities are reverted by divalent cations.<sup>8,128,130</sup> Although in most reported cases, expression of the two types of peptides are restricted to the seeds,<sup>135,137,138</sup> hevein itself and some hevein-like peptides have been found in other tissues.<sup>134,136</sup> Transgenic overexpression in tobacco plants of hevein- and knottin-like peptides did not result in enhanced resistance to the fungus *Alternaria longipes*,<sup>139</sup> although transgenic tomato fruits expressing a hevein peptide were less susceptible than control plants to infection by the opportunistic fungus *Trichoderma hamatum*.<sup>140</sup>

#### Four-Cysteine Antimicrobial Peptides

At least two families of antimicrobial peptides with four cysteines have been reported: the MBP-1, 33-residue peptide from maize,<sup>141</sup> and a group of 20-residue peptides (Ib-AMPs) isolated from the seeds of *Impatiens balsamina*.<sup>142,143</sup> The first peptide is active against fungi, as well as against gram-positive and gram-negative bacteria,<sup>141</sup> whereas the Ib-AMPs inhibit fungi and gram-positive bacteria.<sup>142</sup> The structure of Ib-AMPs has been recently investigated by CD and two-dimensional proton nmr.<sup>143</sup> Mature Ib-AMPs are generated by processing of multipetide precursors<sup>142</sup> in a similar manner as the apidaecins, which are antimicrobial peptides from the honey bee.<sup>144,145</sup>

#### Twelve-Cysteine Peptides

A new type of antimicrobial peptide with twelve cysteines, snak-in-1 (St-SN1), has been recently dis-

covered in potato tubers.<sup>146</sup> The peptide, which is 63-residue long, is active at  $<10 \mu\text{M}$  concentrations against fungi, gram-positive and gram-negative bacteria, is able to aggregate bacteria, and does not have any effect on artificial lipid membranes.<sup>53,146</sup> Although no other member of this peptide family has been isolated yet, its homologues must be ubiquitous, as judged from the multiplicity of homologous cDNAs that have been reported.<sup>146</sup> Snakin-sensitive mutants of the bacterium *Erwinia chrysanthemi* were much less virulent than the wild type in potato tubers, where snak-in is the major antimicrobial peptide.<sup>147</sup> This observation greatly supports a defense role for this protein family.

### FINAL CONSIDERATIONS

As judged from the evidence reviewed here, the role of antimicrobial peptides in the "innate immunity" system of plants seems to be well established. These peptides are part of developmentally regulated, pre-existing defense barriers, and/or may be accumulated as a result of the induction of the corresponding genes upon infection.

Two lines of evidence are particularly relevant in the demonstration of an important role of antimicrobial peptides in plant defense: overexpression of some peptides enhance plant tolerance to pathogens<sup>62,63,78,123,139</sup> and peptide-sensitive mutants of the pathogens show significantly decreased virulence toward plant tissues in which these peptides are present.<sup>65,147</sup> Furthermore, the latter type of evidence indicates that both plant and animal pathogens deal in a similar way with host defenses, as the equivalent mutants of animal pathogens also show decreased virulence.<sup>147,148</sup> The possibility that the pathogen defense system against antimicrobial peptides may show specificity toward the peptide type has been suggested,<sup>147</sup> and might be highly relevant in plant-pathogen interactions.

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